A Role for Histone H2B During Repair of UV-Induced DNA Damage in *Saccharomyces cerevisiae*

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ABSTRACT

To investigate the role of the nucleosome during repair of DNA damage in yeast, we screened for histone H2B mutants that were sensitive to UV irradiation. We have isolated a new mutant, *htb1-3*, that shows preferential sensitivity to UV-C. There is no detectable difference in bulk chromatin structure or in the number of UV-induced cis-syn cyclobutane pyrimidine dimers (CPD) between *HTB1* and *htb1-3* strains. These results suggest a specific effect of this histone H2B mutation in UV-induced DNA repair processes rather than a global effect on chromatin structure. We analyzed the UV sensitivity of double mutants that contained the *htb1-3* mutation and mutations in genes from each of the three epistasis groups of *RAD* genes. The *htb1-3* mutation enhanced UV-induced cell killing in *rad1Δ* and *rad52Δ* mutants but not in *rad6Δ* or *rad18Δ* mutants, which are defective in postreplicational DNA repair (PRR). When combined with other mutations that affect PRR, the histone mutation increased the UV sensitivity of strains with defects in either the error-prone (*rev1Δ*) or error-free (*rad30Δ*) branches of PRR, but did not enhance the UV sensitivity of a strain with a *rad5Δ* mutation. When combined with a *ubc13Δ* mutation, which is also epistatic with *rad5Δ*, the *htb1-3* mutation enhanced UV-induced cell killing. These results suggest that histone H2B acts in a novel *RAD5*-dependent branch of PRR.

THE structure of chromatin is intimately linked to the function of the eukaryotic genome. The basic repeating unit of chromatin, the nucleosome, is assembled in a two-step process in which a tetramer of histones H3 and H4 is first deposited onto DNA, followed by the association of two H2A-H2B heterodimers (for review, see Wolffe 1998). Subsequent folding of nucleosome arrays then leads to multiple levels of chromatin compaction (Hayes and Hansen 2001). Chromatin is generally considered to present a barrier to processes that occur on DNA, and numerous studies have shown that events in transcription can be inhibited by the presence of nucleosomes. The repair of DNA damage also occurs in a chromatin context, but unlike transcription, the role of nucleosomes during repair processes remains less clear. However, a number of studies have revealed a mutual influence between chromatin structure and DNA repair, with the packaging of DNA into chromatin affecting both the acquisition as well as the repair of lesions induced by UV irradiation (for review, see Smerdon and Thoma 1998). Cis-syn cyclobutane pyrimidine dimers (CPDs) are formed around the dyad axis of the nucleosome and at sites where the minor groove of the DNA superhelix faces the histone octamer less frequently than in linker DNA (Liu *et al.* 2000). In addition, various steps in nucleotide excision repair (NER) are significantly inhibited by the presence of nucleosomes (Thoma 1999; Hara *et al.* 2000; Liu and Smerdon 2000).

During the process of transcriptional activation, chromatin is frequently remodeled and/or covalently modified through the activity of evolutionarily conserved remodeling factors (Kornberg and Lorch 1999; Travers 1999; Tyler and Kadonaga 1999). Recent studies suggest that activities similar to those used in transcription may also facilitate DNA repair in chromatin (for review, see Meijer and Smerdon 1999). In mammalian cells, both ATP-dependent nucleosome remodeling factors and histone-modifying enzymes have been associated with the increased accessibility of chromatin templates during NER (Brand *et al.* 2001; Ura *et al.* 2001). In *Saccharomyces cerevisiae*, mutations in the ATP-dependent nucleosome remodeling complex, Ino80, confer hypersensitivity to a wide range of DNA-damaging agents, suggesting that this complex plays a direct role in altering chromatin structure during DNA repair (Shen *et al.* 2000).

Besides nucleosome remodeling factors, two evolutionarily conserved chromatin assembly factors, antisilencing factor 1 (ASF1) and chromatin assembly factor 1 (CAF-I), have also been implicated in DNA repair. In yeast, mutations in ASF1 cause hypersensitivity to double-strand breaks (DSBs), but not to UV irradiation, while CAF-I mutations confer UV sensitivity in preference to other types of damage (Kaufman *et al.* 1997;

Game and Kaufman 1999; Emili *et al.* 2001; Hu *et al.* 2001). Both factors assemble acetylated forms of histones H3 and H4 into nucleosomes during DNA replication, raising the possibility that they perform a similar role on newly repaired DNA (Smith and Stillman 1991b; Verreault *et al.* 1996). Consistent with this view, CAF-I is recruited onto DNA after UV irradiation of human cells and promotes extensive nucleosome assembly during the repair of a damaged template *in vitro* (Gaillard *et al.* 1996; Martini *et al.* 1998). These data suggest that nucleosomes are disassembled in the vicinity of DNA damage.

In eukaryotes, various types of DNA damage are repaired by specific mechanisms. On the basis of genetic epistasis analysis, the genes of *S. cerevisiae* that confer resistance to DNA-damaging agents have been assigned to three major groups (for reviews, see Friedberg *et al.* 1995; Game 2000). The *RAD3* group controls NER, which is responsible for the excision of UV-induced pyrimidine dimers or other bulky adducts. The *RAD52* group repairs double-strand breaks induced by ionizing radiation and other kinds of damage and mediates homologous recombination. Genes in the third group, which have more complex roles and are less well understood, show an epistatic relationship with *RAD6* (for review, see Kunz *et al.* 2000). The *RAD6* group repairs or bypasses multiple forms of DNA lesions during or after DNA synthesis and contains genes whose products function in DNA replication and protein ubiquitylation (Lawrence and Christensen 1976; Prakash 1981; Sung *et al.* 1988; Liefshitz *et al.* 1998). Genetic epistasis studies have also placed CAF-I in the *RAD6*-dependent postreplication repair (PRR) pathway, suggesting a role for chromatin assembly in this pathway (Game and Kaufman 1999).

In this study, we focused on the role of histone H2B in the repair of UV-induced DNA damage. The notion that individual histones play specific roles in DNA damage repair is supported by the observation that double-strand breaks in both human and yeast cells induce the phosphorylation of the C terminus of H2A (the H2A variant H2A.X in humans and the major H2A-1/H2A-2 isoforms in yeast; Rogakou *et al.* 1998; Chen *et al.* 2000; Downs *et al.* 2000; Paull *et al.* 2000). Modification of this histone occurs rapidly in regions surrounding DSBs in human cells, suggesting that it might help to disrupt chromatin at these sites either by directly altering H2A-DNA interactions or by recruiting chromatin remodeling factors (Rogakou *et al.* 1998; Paull *et al.* 2000). Its role in chromatin disruption is supported by the observation that a yeast strain containing a mutation that mimics the phosphorylated form of H2A shows extensive nucleosome instability (Downs *et al.* 2000). Since H2B-DNA interactions play an important role in stabilizing the nucleosome (Luger *et al.* 1997a,b; White *et al.* 2001), we reasoned that H2B might also play a specific role in the modulation of chromatin structure

during DNA repair. We have isolated a new mutant of histone H2B, *htb1-3*, which shows sensitivity to UV irradiation in preference to other genotoxic agents. The UV sensitivity of this mutant does not result from an increase in the number of CPD lesions formed by UV-C or from a global defect in chromatin stability. Genetic epistasis analysis showed that the H2B mutation affected the *RAD6/RAD18*-dependent PRR pathway and specifically a novel, *RAD5*-dependent sub-branch of this pathway. *RAD5* encodes a RING finger protein with homology to the *SNF2* family of ATPases, which have known roles in nucleosome destabilization (Hirschhorn *et al.* 1992; Johnson *et al.* 1992; Pollard and Peterson 1998). Previous studies have linked the activity of Rad5p to chromatin (Ulrich and Jentsch 2000), and our results suggest a role for histone H2B in its activity.

MATERIALS AND METHODS

Yeast strains and media: The *S. cerevisiae* strains used in this study are listed in Table 1 and are isogenic to a W303 strain in which the *rad5-535* allele had been corrected to wild type (obtained from H. Klein). Each strain was derived from JR5-2A, which carries the frameshift alleles *htb1-1* and *htb2-1* and plasmid YCp50-HTB1 (Recht and Osley 1999). *RAD* genes were disrupted in strain JR5-2A or EM1 by transformation with linear fragments isolated from plasmids that contained marked deletion constructs: pHU249, *ubc13::HIS3* (*Sac*I-*Apa*I); pTW033, *rad9::HIS3* (*Not*I); pSH87, *rad5::URA3* (*Hind*III-*Eco*RI); pR30-2, *rad30::URA3* (*Eco*RI); pREV1.6, *rev1Δ::URA3* (*Sph*I); pR18.119, *rad18Δ::LEU2* (*Hind*III-*Bam*HI); pL962, *rad1Δ::LEU2* (*Hind*III); pSM20, *rad52Δ::LEU2* (*Bam*HI); p46, *rad6Δ::hisG::URA3::hisG* (*Bam*HI); pPK102, *cac1Δ::hisG::URA3::hisG* (*Bam*HI). The presence of the disruptions was confirmed by assaying for expected levels of sensitivity to UV irradiation and other genotoxic agents and in several cases by rescue with a plasmid carrying the wild-type allele. Standard protocols were followed for preparation of yeast media and transformation (Adams *et al.* 1997).

Plasmids: Plasmids YCp50-HTB1 and pRS314-HTB1 have been described (Recht and Osley 1999). Both plasmids carry the *HTB1* open reading frame (ORF) as a *Bst*EII-*Not*I fragment under control of the wild-type *HTA1*-*HTB1* promoter. pRS314ΔNotI-HTB1 was derived from pRS314-HTB1 by removal of a *Not*I-*Bam*HI restriction fragment from the polylinker in pRS314. pRS314-htb1-3 was generated by targeting mutations to the *HTB1* ORF in pRS314-HTB1 in two steps using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The following mutagenic primers were used: V47F, 5′-CTCTTCTTACATTTACAAATTTTTGAAGC AAACTCACCC-3′ and 5′-GGGTGAGTTTGCTTCAAAAATT TGTAAATGTAAGAAGAG-3′; and Y86F-N87S, 5′-CTAAATTG GCTGCGTTTAGCAAGAAGTCTACTATC-3′ and 5′-GATAGT AGACTTCTTGCTAAACGCAGCCAATTTAG-3′. Construction of Flag epitope-tagged pRS314-HTB1 has been described (Recht and Osley 1999). Flag epitope-tagged pRS314-htb1-3 was obtained by targeting the V47F, Y86F, N87S mutations to Flag-tagged *HTB1* in pRS314. The presence of the mutations was confirmed by DNA sequence analysis. pRS324ΔNotI-HTB1 was obtained by inserting a *Not*I-*Bam*HI fragment from pRS314-HTB1 into *Not*I-*Bam*HI-digested plasmid pRS324ΔNotI.

Mutagenesis of *HTB1*: To obtain UV-sensitive *htb1* alleles, we adapted a method that is based on the low fidelity of Taq DNA polymerase (Hirschhorn *et al.* 1995). The *HTB1* ORF

TABLE 1

S. cerevisiae strains

Strain	Genotype	Source
JR5-2A	*MAT**a** htb1-1 htb2-1 ura3-1 leu2-3,-112 ade2-1 trp1-1 his3-11,-15 can1-100 ssd1 <pRS314-HTB1 or pRS314-htb1-3>*	J. Recht
EM1	*MAT**a** htb1-1 htb2-1 ura3-1 leu2-3,-112::LEU2 ade2-1 trp1-1 his3-11,-15 can1-100 ssd1 <Ycp50-HTB1 or pRS314-HTB1>*	This study
EM4-d1	*MAT**a**/MATα htb1-1/htb1-1 htb2-1/htb2-1 ura3-1/ura3-1 leu2-3,-112/leu2-3,-112::LEU2 ade2-1/ade2-1 trp1-1/trp1-1 his3-11,-15/his3-11,-15 can1-100/can1-100 ssd1/ssd1 <pRS314-HTB1 or pRS314-htb1-3>*	This study
EM81	*MAT**a** htb1-1 htb2-1 bar1Δ::hisG rad9::HIS3 ura3-1 leu2-3,-112::LEU2 ade2-1 trp1-1 his3-11,-15 can1-100 ssd1 <Ycp50-HTB1 or pRS314-htb1-3>*	This study
EM83	*MAT**a** htb1-1 htb2-1 cac1Δ::hisG ura3-1 leu2-3,-112::LEU2 ade2-1 trp1-1 his3-11,-15 can1-100 ssd1 <pRS314-HTB1 or pRS413-htb1-3>*	This study
EM84	*MAT**a** htb1-1 htb2-1 rad18Δ::LEU2 ura3-1 leu2-3,-112 ade2-1 trp1-1 his3-11,-15 can1-100 ssd1 <pRS314-HTB1 or pRS413-htb1-3>*	This study
EM86	*MAT**a** htb1-1 htb2-1 rad1Δ::LEU2 ura3-1 leu2-3,-112 ade2-1 trp1-1 his3-11,-15 can1-100 ssd1 <YCp50-HTB1 or pRS314-htb1-3>*	This study
EM87	*MAT**a** htb1-1 htb2-1 rad52Δ::LEU2 ura3-1 leu2-3,-112 ade2-1 trp1-1 his3-11,-15 can1-100 ssd1 <YCp50-HTB1 or pRS314-htb1-3>*	This study
EM90	*MAT**a** htb1-1 htb2-1 rad6Δ::hisG ura3-1 leu2-3,-112 ade2-1 trp1-1 his3-11,-15 can1-100 ssd1 <pRS314-HTB1 or pRS413-htb1-3>*	This study
EM92	*MAT**a** htb1-1 htb2-1 rad5Δ::URA3 leu2-3,-112 ura3-1 ade2-1 trp1-1 his3-11,-15 can1-100 ssd1 <pRS314-HTB1 or pRS413-htb1-3>*	This study
EM94	*MAT**a** htb1-1 htb2-1 rad30Δ::hisG-URA3-hisG leu2-3,-112 ura3-1 ade2-1 trp1-1 his3-11,-15 can1-100 ssd1 <pRS413-HTB1 or pRS314-htb1-3>*	This study
EM95	*MAT**a** htb1-1 htb2-1 rev1Δ::URA3 leu2-3,-112 ura3-1 ade2-1 trp1-1 his3-11,-15 can1-100 ssd1 <pRS413-HTB1 or pRS314-htb1-3>*	This study
EM96	*MAT**a** htb1-1 htb2-1 ubc13Δ::HIS3 leu2-3,-112::LEU2 ura3-1 ade2-1 trp1-1 his3-11,-15 can1-100 ssd1 <YCp50-HTB1 or pRS314-htb1-3>*	This study

was amplified from pRS314-*HTB1* using primers to generate a PCR product extending from 116 bp upstream of the *HTB1* ATG (5′-CTCAGATGGTCAGATTTATTA-3′) to 147 bp downstream of the *HTB1* termination codon (5′-ATTTCGAGAACA CAATTTTAC-3′). PCR reactions were performed with 100 ng of plasmid DNA in the presence of 0.25 mM $MnCl_2$ and 7.5 mM $MgCl_2$ to generate an average of five to seven base changes per *HTB1* ORF. The PCR products were cotransformed into strain JR5-2A [Ycp50-*HTB1*] along with gel-purified plasmid pRS314-*HTB1* that had been digested with *Bst*EII-*Not*I to release the *HTB1* ORF. Following gap repair *in vivo* (Hirsch-horn *et al.* 1995), Trp$^+$ Ura$^+$ transformants were selected. The transformants were then patched onto 5-fluoroorotic acid plates to identify viable cells that had lost plasmid YCp50-*HTB1*. Trp$^+$ Ura$^-$ cells were screened for hypersensitivity to UV irradiation by exposure to 100 J/m^2 of UV-C.

Measurement of 2μ plasmid DNA topoisomers: DNA was isolated from 10-ml YPD cultures grown to midlog as described (Morse 1999). Total DNA was electrophoresed through a 0.7% agarose gel in Tris-phosphate buffer (0.09 M Tris-phosphate, 0.002 M EDTA) containing 10 μg/ml of chloroquine at 50 V for 27 hr at 4°. DNA was transferred to Hybond C Extra membrane (Amersham, Piscataway, NJ), and plasmid DNA topoisomers were detected by hybridization to a 687-bp *Ava*I-*Xba*I fragment of 2μ DNA that was labeled by random priming. DNA topoisomer distributions were quantified and analyzed using Quantification One software from Bio-Rad (Richmond, CA).

Micrococcal nuclease digestion of chromatin: Nuclei were isolated according to the method of Bernardi *et al.* (1991). Spheroplasts were prepared from 500 ml of cells grown to midlog phase in YPD following treatment with 0.5 mg/ml Zymolyase in 1 M sorbitol. Spheroplasts were lysed in 18% Ficoll, 20 mM potassium phosphate, 1 mM $MgCl_2$, 0.25 mM EGTA, 0.25 mM EDTA pH 6.8, 1 mM phenylmethylsulfonyl fluoride (PMSF) and resuspended in 7 ml of 10 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM KCl, 1 mM EDTA, and 1 mM PMSF. Aliquots of isolated nuclei were immediately subjected to micrococcal nuclease (MNase) digestion with indicated amounts of MNase in the presence of 5 mM $CaCl_2$ for 5 min at 37°. DNA was purified by proteinase K treatment, phenol-chloroform extraction, and ethanol precipitation. DNA was subjected to electrophoresis through a 1.5% agarose gel in Tris-borate buffer (0.09 M Tris-borate, 0.001 M EDTA). The MNase profile of total genomic DNA was visualized by ethidium bromide staining, and DNA was then transferred to Hybond C Extra membrane. The MNase profiles of the *SUC2* and *HIS3* loci were measured by hybridization to a *SUC2* probe fragment obtained by PCR (+133 to +770) or to a 1.8-kb *HIS3 Bam*HI fragment that contained the *HIS3* ORF + 5′ flanking sequences, both labeled by random priming.

Measurement of CPD lesions: Twenty-milliliter cultures of *HTB1* and *htb1-3* strains were grown to midlog phase in YPD, washed with water, and resuspended in 30 ml of phosphate-buffered saline (PBS). Three 8.5-cm diameter petri dishes containing 10 ml of the cell suspension were kept on ice and irradiated with 0, 30, or 150 J/m^2 of UV-C. Six milliliters of UV-irradiated or control cells were then incubated in 50% ethanol plus 12 mM EDTA. DNA was extracted and purified as described (Adams *et al.* 1997) and quantified using a DyNA Quant fluorometer (Pharmacia Biotech, Piscataway, NJ). For each sample, aliquots of 10 and 15 ng were adjusted to a

final volume of 400 µl and treated as described (Bio-Dot user manual, Bio-Rad), and 200 µl were then slot-blotted in duplicate onto a 0.2-µm nitrocellulose membrane (PROTRAN, BA83; Schleicher & Schuell, Keene, NH). To detect CPDs, the blot was blocked overnight in 0.5% nonfat milk in TBS-Tween (136 mm NaCl, 2.7 mm KCl, 25 mm Tris-Cl pH 8.0 plus 0.1% Tween 20) and then incubated with anti-CPD antibodies (1:250 dilution of clone KTM53; Kamiya Biomedical, Thousand Oaks, CA) in 0.5% TBS, 0.02% Tween 20 for 1 hr at 37° (PERDIZ *et al.* 2000). Enhanced chemiluminescence was used to detect antigen-antibody complexes according to the manufacturer's directions (Amersham). The same blot was also hybridized to a probe obtained by random priming of bulk genomic DNA extracted from strain EM1. The number of CPD lesions was normalized to the amount of DNA present on the filters using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

DNA damage sensitivity assays: Exponentially growing cultures of wild-type and mutant strains were grown in YPD to $\sim 6 \times 10^6$ cells/ml. UV survival was measured after spreading appropriate dilutions of the cultures in duplicate on YPD plates and then subjecting the plates to specific UV doses at 254 nm (Spectroline XX-15G lamp; Spectronics, Westbury, NY). Plates were incubated in the dark for 3 days at 30° and the number of colonies was counted. Each experiment presented here was repeated at least three times, and data from single representative experiments are shown. For each experiment, data points are the average of four determinations, and error bars represent the range of values (minimum and maximum) obtained in that experiment.

Bleomycin sensitivity was measured by spotting 3 µl of 10-fold serial dilutions of cells onto YPD plates containing 6 milliunits/ml bleomycin, followed by incubation for 2 days at 30°. Sensitivity to gamma irradiation was measured by harvesting 5 ml of culture, resuspending the cells in 1 ml of ice-cold PBS in a 1.5-ml Eppendorf tube, and then irradiating with 150 Gy of gamma irradiation using a ^{137}Cs source (Mark 1, model 68; J. L. Shepherd & Associates, San Fernando, CA). After irradiation, cells were resuspended in 5 ml of water and 3 µl of 10-fold serial dilutions were spotted onto YPD plates. The plates were incubated at 30° for 2 days before counting survivors.

UV-induced mutagenesis: Exponentially growing cells were grown in YPD to ~ 6–10×10^6 cells/ml, washed, and resuspended in distilled water at a density of 5–10×10^7 cells/ml. Cells were plated in duplicate on YPD plates to determine viable cell number and on synthetic media lacking adenine or tryptophan to measure reversion of *ade2-1* or *trp1-1*. After UV irradiation at doses of 2, 10, and 50 J/m², the plates were incubated in the dark at 30°. Survival was measured after 3 days on YPD plates and reversion frequency was measured after 6 days on synthetic media.

RESULTS

Isolation of a new class of H2B mutants that are sensitive to UV irradiation: In *S. cerevisiae*, histone H2B is encoded by two unlinked genes, *HTB1* and *HTB2*, which together are essential for cell viability (for review, see OSLEY 1991). We used a strain that carried frameshift mutations in the genomic copies of both *HTB* genes and whose viability was maintained by a *URA3/ CEN* plasmid that contained the *HTB1* gene as the only source of H2B in the cell (RECHT and OSLEY 1999). Using a PCR-based mutagenesis procedure that reduced
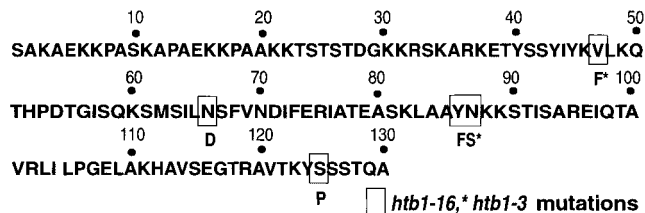

FIGURE 1.—Location of the *htb1-16* and *htb1-3* mutations in histone H2B. The locations of the five amino acid changes in *htb1-16* and three amino acid changes in *htb1-3* are shown on the sequence of the *HTB1* ORF. The *htb1-3* mutations change residues that fall in loop 1 (V47F) and loop 2 (Y86F, N87S) of H2B. These two structural domains are involved in the binding of DNA on the surface of the histone octamer.

the fidelity of Taq DNA polymerase (HIRSCHHORN *et al.* 1995), we generated random mutations in the *HTB1* ORF *in vitro* and screened *in vivo* for viable mutants that were hypersensitive to killing by UV-C. From ~ 700 colonies, we identified four recessive *htb1* mutants that showed a similar hypersensitivity to UV irradiation (Figure 1 and data not shown). The UV sensitivity was moderate in comparison to the sensitivity exhibited by many of the known *rad* mutants.

DNA sequence analysis revealed that multiple mutations were present in the H2B ORF of each UV-sensitive mutant. A comparison of the amino acid changes did not identify residues that were commonly mutated in all four *htb1* alleles, and we therefore focused on one mutant, *htb1-16*, which encoded five altered amino acids (V47F, N66D, Y86F, N87S, and S127P). None of the five mutations on its own was sufficient to confer UV sensitivity (data not shown), and only combination of the mutations V47F, Y86F, and N87S recapitulated the phenotype of the original *htb1-16* mutant (Figure 1 and data not shown). Our subsequent studies utilized the mutant that contained these three changes, which we named *htb1-3*. Besides exhibiting UV sensitivity, this mutant, like *htb1-16*, also showed poor growth on YPD plates at 16° (data not shown).

We compared the UV sensitivity of strains carrying the *htb1-3* mutation on either a *CEN* or multicopy plasmid. In both cases, similar levels of cell survival were seen after UV irradiation (Figure 2). These results suggested that the UV sensitivity of the *htb1-3* mutant was not caused by a reduction in the cellular levels of histone H2B. In support of this conclusion, Western blot analysis of Flag epitope-tagged H2B isolated from a wild-type strain and the *htb1-3* mutant revealed no significant differences in the levels of this histone (data not shown).

Chromatin structure in the *htb1-3* mutant: Many forms of DNA lesions are preferentially targeted to linker DNA between nucleosomes, reflecting the greater accessibility of these regions to DNA damage (KUO and HSU 1978; CONCONI *et al.* 1984; and for reviews see SMERDON and THOMA 1998; THOMA 1999). Thus, it was possible that the increased UV sensitivity of the *htb1-3* mutant was
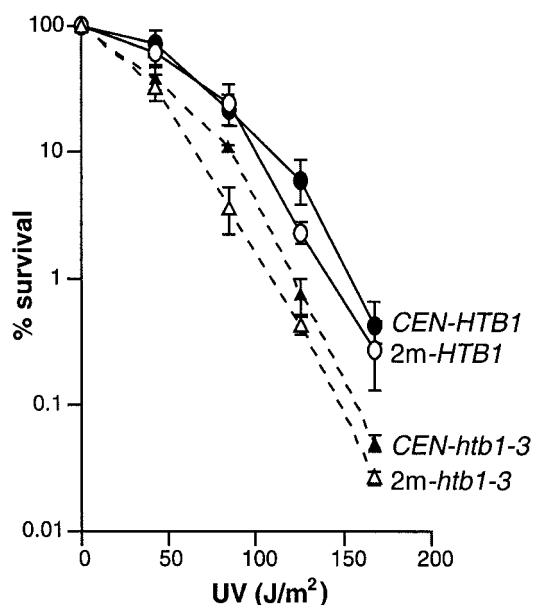
FIGURE 2.—UV sensitivity of the *htb1-3* mutant. Strain EM1 containing *HTB1* or *htb1-3* carried on a *CEN* or 2μ plasmid was irradiated with UV-C, and survival was measured as a function of UV dose.

due to a global disruption in chromatin structure that effectively increased the number of sites where lesions could occur. To test this possibility, we first examined the pattern of MNase digestion of bulk chromatin in strains carrying either a wild-type *HTB1* gene or the *htb1-3* allele. MNase preferentially cleaves chromatin in the linker regions between nucleosomes and thereby provides an indication of both the presence and spacing of nucleosomes in the chromatin fiber. The two strains showed no significant differences in the pattern of MNase digestion of bulk chromatin or of two specific loci, *HIS3* and *SUC2* (Figure 3). Next, we examined the ability of nucleosomes to supercoil DNA *in vivo* by analyzing the distribution of DNA topoisomers from the endogenous 2μ plasmid. Each time a nucleosome is assembled onto a closed circular DNA molecule, a single superhelical turn is introduced (WORCEL *et al.* 1981). If the *htb1-3* mutation led to nucleosome instability or loss, then we would expect to see a shift in the distribution of plasmid DNA topoisomers as analyzed by chloroquine-agarose gel electrophoresis. Again, no apparent differences were detected between the two strains (Figure 4). Together, the results suggest that the *htb1-3* mutation does not grossly disrupt bulk chromatin structure, although we cannot exclude the possibility that the mutation causes locus-specific alterations of chromatin.

Induction of cyclobutane pyrimidine dimers is not increased in the *htb1-3* mutant: UV irradiation induces various kinds of lesions, the most abundant being CPDs (for review, see FRIEDBERG *et al.* 1995). To examine the effect of the *htb1-3* allele on CPD formation, we measured the number of lesions induced in bulk geno-

mic DNA immediately after UV irradiation. Using a Western blot analysis with monoclonal antibodies specific for CPDs (PERDIZ *et al.* 2000), we detected a UV-dependent increase in the number of CPDs in both *HTB1* and *htb1-3* strains (Figure 5). The anti-CPD blots were scanned and the results were normalized to the amount of DNA present on the filters (MATERIALS AND METHODS). The results from three independent experiments indicated that there was no apparent difference in the number of CPDs present in wild-type and mutant strains (Figure 5 and data not shown). This suggests that the *htb1-3* mutation does not significantly influence induction of CPDs by UV-C.

Sensitivity of the *htb1-3* mutant to other genotoxic agents: Genotoxic agents other than UV irradiation induce additional forms of DNA lesions and lead to repair by alternate pathways. We therefore tested the survival of the *htb1-3* mutant after exposure to ionizing irradiation, methyl methanesulfonate (MMS), or bleomycin (Figure 6 and data not shown). Each of these agents produces a broad spectrum of DNA damage that includes base damage, single-strand breaks (SSBs), and DSBs, lesions that are generally repaired by the *RAD52*-dependent recombinational repair pathway (RESNICK and MARTIN 1976; and for review see NICKOLOFF and HOEKSTRA 1998). Both *htb1-3* haploid and diploid cells behaved like wild-type cells and were resistant to a dose of gamma irradiation that caused >99.9% lethality in a *rad9Δ* mutant, which is hypersensitive to a wide range of DNA-damaging agents (Figure 6, top). Similar results were observed after incubation of both *htb1-3* strains on plates that contained 0.02% MMS (data not shown). These results indicated that the *htb1-3* mutant was not defective in DSB repair. Notably, the *htb1-3* mutant was hypersensitive to bleomycin compared to the wild-type strain (Figure 6, bottom).

Epistasis analysis of the *htb1-3* UV-sensitive phenotype: Many forms of DNA damage are repaired in yeast through the action of genes falling in three broad epistasis groups. To determine if the *htb1-3* mutation affected the function of one of these three groups, we disrupted a gene from each group in both *HTB1* and *htb1-3* cells and compared the UV sensitivity of the double mutants to the corresponding single mutants. We first examined the relationship between *htb1-3* and *rad1Δ*, which is defective in the endonuclease that incises DNA on the 5′ side of lesions during NER (KLEIN 1988; SCHIESTL and PRAKASH 1988; FRIEDBERG *et al.* 1995; NICKOLOFF and HOEKSTRA 1998). Exposure to UV-C enhanced the killing of a *rad1Δ htb1-3* mutant compared to a *rad1Δ* mutant (Figure 7A), indicating that the *htb1-3* mutation affects a pathway other than NER.

The *RAD52* epistasis group is involved in recombinational repair of double-strand breaks induced by agents such as ionizing radiation as well as by high doses of UV-C (RESNICK and MARTIN 1976; MORTENSEN *et al.* 1996; NICKOLOFF and HOEKSTRA 1998). We examined
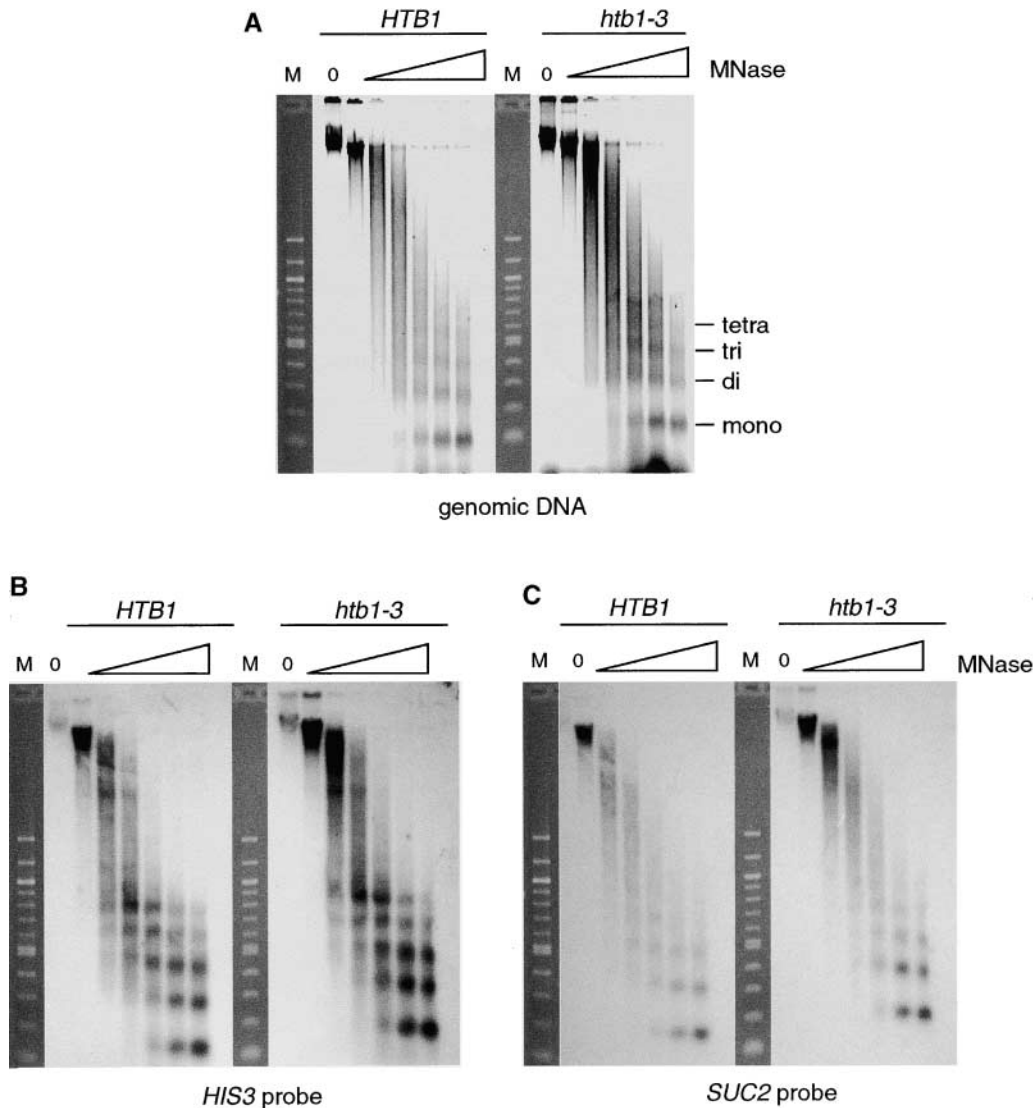
FIGURE 3.—Micrococcal nuclease sensitivity of chromatin isolated from *HTB1* and *htb1-3* strains. Nuclei were prepared from exponential cultures of strain JR5-2A containing pRS314-*HTB1* or pRS314-*htb1-3* and digested with 0, 1, 3, 7, 15, 30, or 50 units of MNase, and purified DNA was submitted to 1.5% agarose gel electrophoresis. (A) Total genomic DNA was visualized by ethidium bromide staining. (B and C) Southern blot analysis was performed using radiolabeled probes that hybridized to (B) *HIS3* or (C) *SUC2*. M corresponds to a 100-bp-molecular-weight marker stained with ethidium bromide, and mono, di, tri, and tetra correspond to multiples of nucleosome size units.

the epistatis relationship between *rad52Δ* and *htb1-3* at doses of UV that give significant killing of *rad52Δ* cells. A *rad52Δ htb1-3* double mutant showed sensitivity to high doses of UV irradiation greater than that of either a *rad52Δ* (Figure 7B) or *htb1-3* (Figure 7D) single mutant. Together with the observation that the *htb1-3* mutant was not hypersensitive to gamma irradiation, this result supports the view that the mutations in H2B do not affect the major pathway of recombinational repair.

The third epistasis group contains a heterogeneous collection of genes involved in PRR. On the basis of double mutant analysis, the most upstream gene in the PRR group is *RAD6*. *RAD6* encodes a multifunctional ubiquitin-conjugating enzyme that targets unknown substrates during the repair of many different types of DNA damage, including damage induced by UV-C (MONTELONE *et al.* 1981; REYNOLDS *et al.* 1985; JENTSCH *et al.* 1987; SUNG *et al.* 1988). A *rad6Δ htb1-3* mutant showed the same sensitivity to UV-C as a *rad6Δ* mutant (Figure 7C), indicating that histone H2B is a member

of the *RAD6* epistasis group. It is a formal possibility that we were unable to detect the effect of the H2B mutations because of the extreme UV sensitivity of *rad6Δ* mutants. However, we consider this unlikely because a *rad1Δ* mutant is as UV sensitive as a *rad6Δ* mutant, and we were able to detect enhanced killing of a *rad1Δ htb1-3* mutant compared to a *rad1Δ* strain (compare Figure 7A and 7C). The assignment of H2B to the *RAD6* epistasis group is further supported by the finding that a *rad18Δ htb1-3* mutant was no more UV sensitive than a *rad18Δ* mutant (Figure 7C). *RAD18* and *RAD6* encode gene products that appear to function upstream of all other PRR functions. Rad18p has been shown to interact directly with Rad6p both *in vivo* and *in vitro* and has been proposed to target Rad6p to sites of DNA damage (CASSIER-CHAUVAT and FABRE 1991; BAILLY *et al.* 1994; ULRICH and JENTSCH 2000). Together, the results indicate that histone H2B plays a role in *RAD6/RAD18*-dependent PRR.

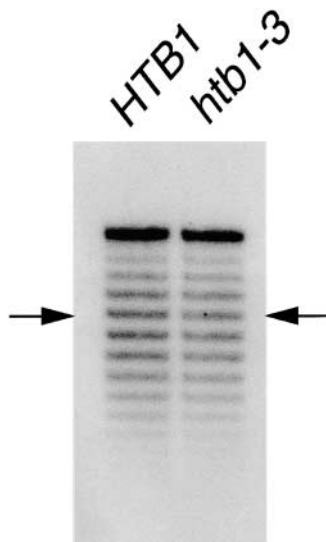**The *htb1-3* mutation affects a *RAD5*-dependent repair**

FIGURE 4.—Effect of the *htb1-3* mutation on the topology of 2μ plasmid DNA. DNA was isolated from strain JR5-2A containing pRS314-*HTB1* or pRS314-*htb1-3* and subjected to electrophoresis through a 0.7% agarose gel containing 10 μg/ml of chloroquine. The distribution of 2μ plasmid DNA topoisomers was measured by Southern blot analysis with a radiolabeled 2μ DNA probe. The arrows indicate the centers of the topoisomer distributions.

pathway: The PRR pathway is perhaps the least understood of the major DNA repair pathways. At least two subpathways of PRR can be distinguished on the basis of whether mutations are generated during the repair process itself (XIAO *et al.* 2000). The error-prone PRR pathway includes the products of the *REV1*, *REV3*, and *REV7* genes, which perform mutagenic translesion synthesis (LAWRENCE and CHRISTENSEN 1976; LAWRENCE *et al.* 1984, 1985; LARIMER *et al.* 1989; BAYNTON *et al.* 1999). The error-free PRR pathway has less effect on damage-induced mutagenesis and has been proposed to contain two different sub-branches, defined by the *RAD5* and *RAD30* genes (JOHNSON *et al.* 1992, 1994, 1999; MCDONALD *et al.* 1997; ROUSH *et al.* 1998). To determine if the *htb1-3* mutation affected the error-prone or error-free PRR pathway, we examined the UV sensitivity of *rev1Δ htb1-3*, *rad5Δ htb1-3*, and *rad30Δ htb1-3* double mutants (Figure 8). Like the *htb1-3* mutant, *rad30Δ* and *rev1Δ* mutants are moderately sensitive to UV. However, both *rev1Δ htb1-3* (Figure 8B) and *rad30Δ htb1-3* (Figure 8C) double mutants exhibited a synergistic reduction in cell survival after UV irradiation. In contrast, the *htb1-3* mutation did not enhance UV killing in a *rad5Δ* mutant (Figure 8A) or in a *rad5Δ rad30Δ* double mutant, which shows extreme sensitivity to UV (data not shown). Together, the results suggest that *rad5Δ* is epistatic to *htb1-3*, thus placing *HTB1* in a *RAD5*-dependent branch of PRR.

The *UBC13* and *MMS2* genes, whose products form a ubiquitin-conjugating complex, also act in a *RAD5*-dependent branch of PRR (BROOMFIELD *et al.* 1998;
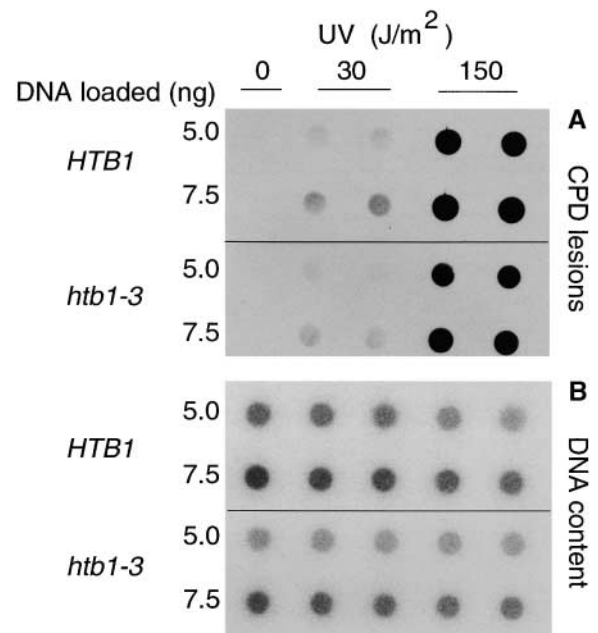

FIGURE 5.—Measurement of CPD lesions in *HTB1* and *htb1-3* strains after UV irradiation. Exponentially growing cultures of strain JR5-2A containing pRS314-*HTB1* or pRS314-*htb1-3* were exposed to 0, 30, or 150 J/m² of UV-C. DNA was extracted, purified, and quantified, and dilutions were spotted onto a nitrocellulose filter. (A) CPD lesions were measured using anti-CPD monoclonal antibodies. (B) The same membrane was hybridized to a bulk genomic DNA probe to normalize the amount of DNA loaded onto the membrane.

HOFMANN and PICKART 1999; BRUSKY *et al.* 2000; ULRICH and JENTSCH 2000). Rad5p is proposed to act upstream of the Ubc13p/Mms2p complex because a *rad5Δ* mutation is epistatic to both the *ubc13Δ* and *mms2Δ* mutations and because Rad5p is required to recruit the Ubc13p/Mms2p complex to chromatin after DNA damage (ULRICH and JENTSCH 2000). To deter-
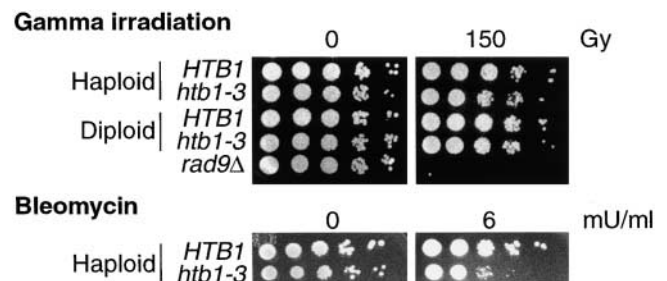

FIGURE 6.—Sensitivity of the *htb1-3* mutant to gamma irradiation and bleomycin. (Top) Exponentially growing cultures of haploid strain EM1, diploid strain EM4-d1, and haploid strain EM81, each containing pRS314-*HTB1* or pRS314-*htb1-3*, were exposed to 0 or 150 Gy of gamma irradiation. Tenfold serial dilutions of cells were spotted onto a YPD plate and incubated for 2 days at 30°. (Bottom) Tenfold serial dilutions of cells from an exponential culture of strain EM1 containing pRS314-*HTB1* or pRS314-*htb1-3* were spotted onto YPD plates containing 0 or 6 milliunits/ml of bleomycin and incubated for 2 days at 30°.
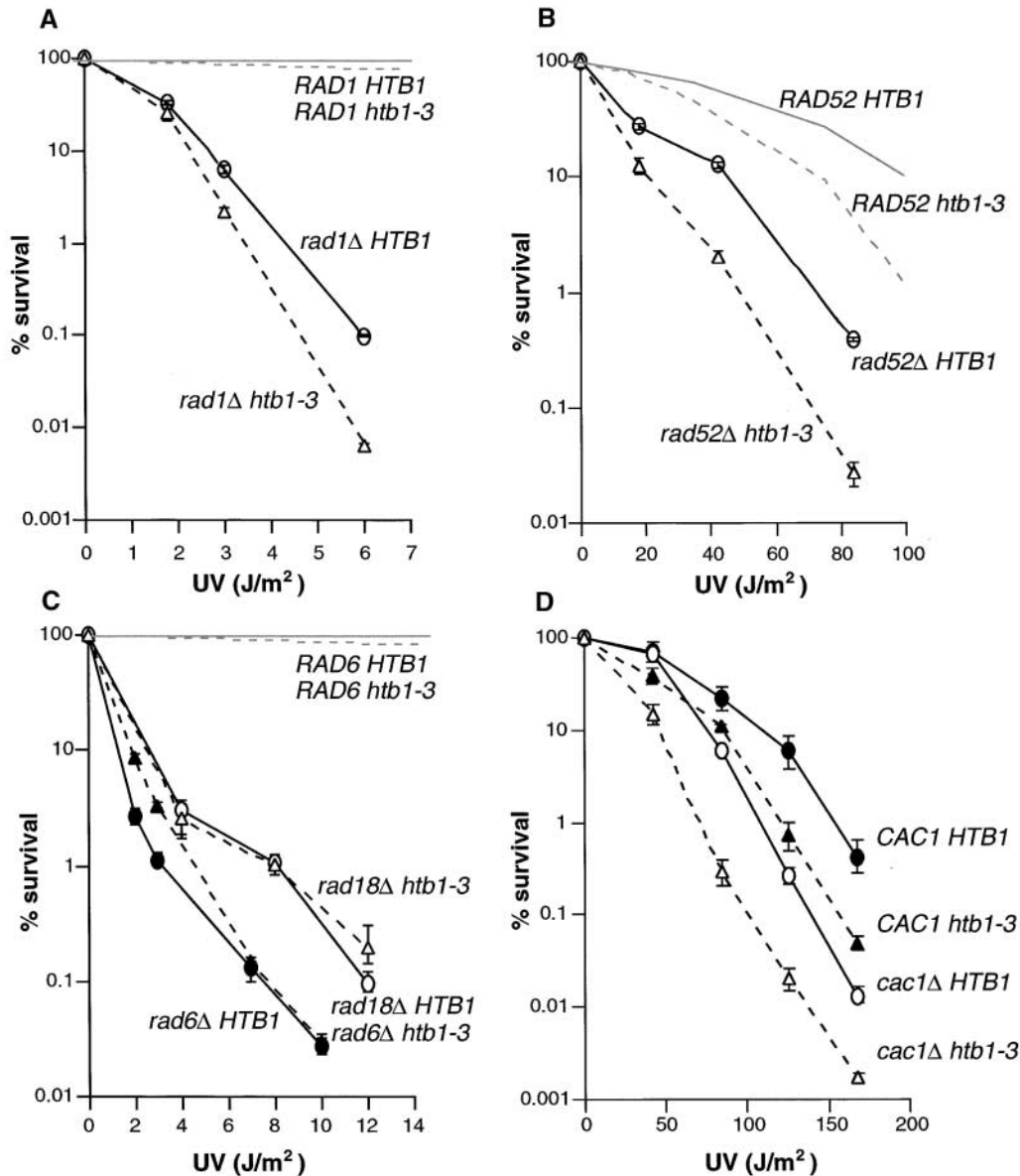
FIGURE 7.—Epistasis analysis of the *htb1-3* UV-sensitive phenotye. Survival after irradiation is plotted as a function of UV dose. (A) NER group (EM86), *rad1Δ* in combination with *htb1-3*; (B) *RAD52* group (EM87), *rad52Δ* in combination with *htb1-3*; (C) *RAD6* group (EM-90; EM84), *rad6Δ* or *rad18Δ* in combination with *htb1-3*; and (D) CAF-I (EM83), *cac1Δ* in combination with *htb1-3*. In A–C, the results from a separate experiment with *HTB1* and *htb1-3* strains are shown for comparison.

mine if the H2B mutations affected this particular activity of Rad5p, we measured the UV sensitivity of a *ubc13Δ htb1-3* mutant (Figure 8D). Cell survival was reduced in the double mutant compared to either single mutant, suggesting that Rad5p-H2B and Rad5p-Ubc13p-Mms2p represent distinct sub-branches of *RAD5*-dependent PRR (see DISCUSSION).

Effect of the *htb1-3* allele on UV-induced mutagenesis: UV-induced DNA damage that is not repaired by NER is bypassed by DNA polymerases that function in either a predominantly error-prone (Rev3/Rev7) or error-free (Rad30) mode (JOHNSON *et al.* 1992; LAWRENCE and HINKLE 1996; MCDONALD *et al.* 1997; ROUSH *et al.* 1998). Thus, mutations in *REV1*, *REV3*, or *REV7* cause a marked reduction in the levels of UV-induced mutations, while mutation of *RAD30* enhances, reduces, or has little effect on UV mutagenesis, depending on the locus examined (MCDONALD *et al.* 1997; ROUSH *et al.* 1998; RAJPAL

et al. 2000). To determine if the *htb1-3* allele affected UV mutagenesis, we measured reversion of the *ade2-1* and *trp1-1* alleles after exposure to different doses of UV-C (Figure 9). The frequency of Ade+ and Trp+ revertants in the histone mutant was similar to that of a wild-type strain. Thus the *htb1-3* allele does not appear to significantly impair UV-induced mutagenesis.

UV sensitivity of a *cac1Δ htb1-3* double mutant: CAF-I is the only other chromatin-associated factor known to participate specifically in the repair of UV-induced DNA damage. This evolutionarily conserved factor deposits histone H3-H4 tetramers onto DNA during both replication and nucleotide excision repair synthesis (SMITH and STILLMAN 1991a,b; KAUFMAN *et al.* 1995; GAILLARD *et al.* 1996; KAMAKAKA *et al.* 1996; MARTINI *et al.* 1998). CAF-I is not essential for cell viability in yeast, but deletion of any one of the three genes that encode its subunits confers moderate sensitivity to UV irradiation
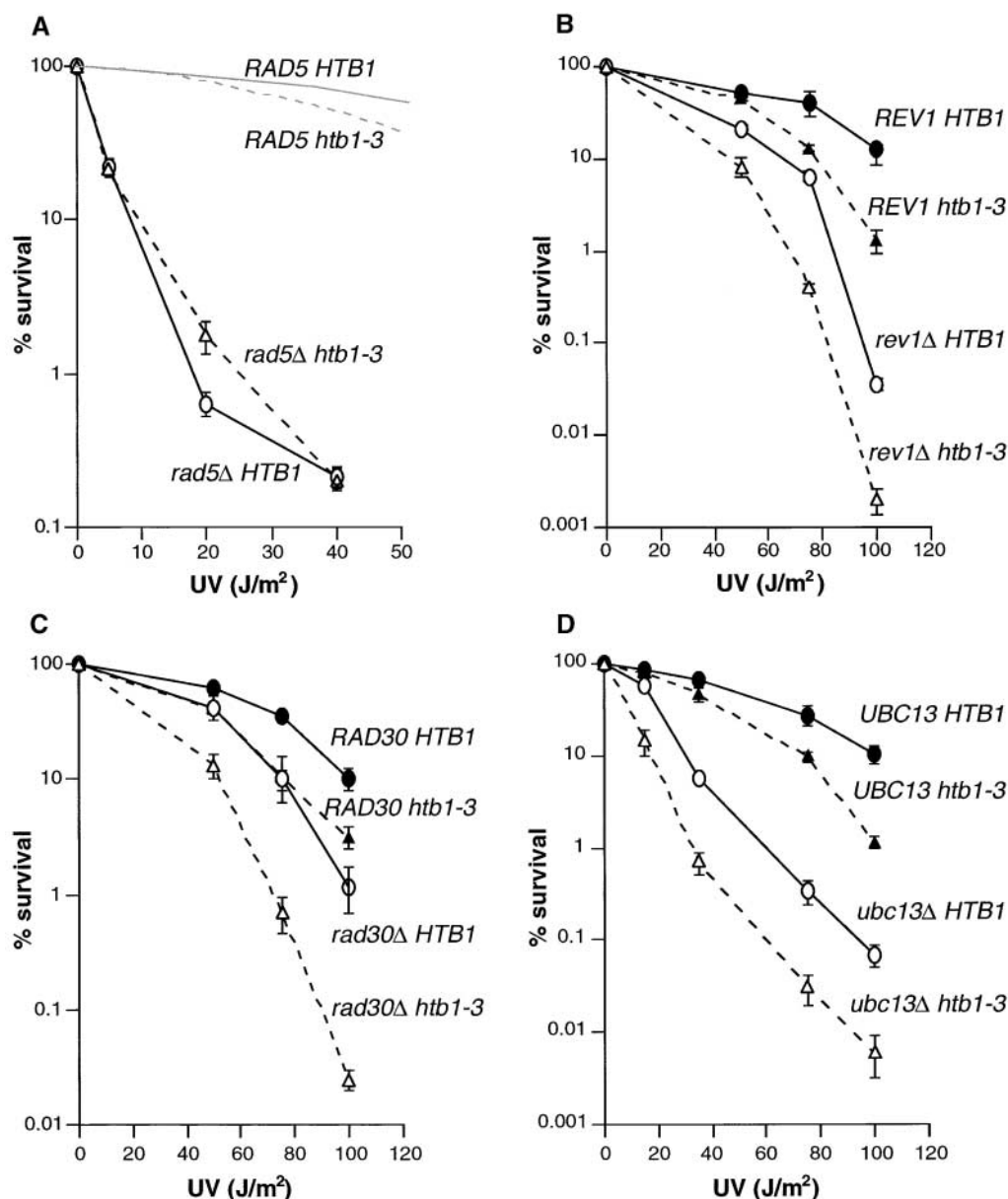
FIGURE 8.—Genetic interactions between *htb1-3* and members of the *RAD6* epistasis group. Survival after irradiation is plotted as a function of UV dose. (A) *RAD5* (EM92), *rad5Δ* in combination with *htb1-3*; (B) *REV1* (EM95), *rev1Δ* in combination with *htb1-3*; (C) *RAD30* (EM94), *rad30Δ* in combination with *htb1-3*; and (D) *UBC13* (EM96), *ubc13Δ* in combination with *htb1-3*. In A, the results from a separate experiment with *HTB1* and *htb1-3* strains are shown for comparison.

(ENOMOTO *et al.* 1997; KAUFMAN *et al.* 1997; GAME and KAUFMAN 1999). Like H2B, CAF-I acts in the error-free pathway of *RAD6*-dependent PRR, but unlike H2B, it appears to function independently of *RAD5* (GAME and KAUFMAN 1999). To determine if H2B plays a role with CAF-I after UV irradiation, we measured survival of a *cac1Δ htb1-3* double mutant following exposure to UV-C (Figure 7D). The double mutant showed reduced survival after UV irradiation compared to either a *cac1Δ* or *htb1-3* mutant, suggesting that H2B and CAF-I act in different branches of PRR.

DISCUSSION

htb1-3, a UV-sensitive mutant of histone H2B: This study describes the characterization of a new mutant of histone H2B that exhibits hypersensitivity to UV irradia-

tion, showing for the first time a link between a specific nucleosome constituent and the repair of UV-induced DNA damage. The UV sensitivity of this mutant results from a combination of three mutations in two structural domains—loop 1 (L1) and loop 2 (L2)—that are common to all four core histones. Both L1 and L2 are involved in the binding of DNA on the surface of the histone octamer (LUGER *et al.* 1997a; WHITE *et al.* 2001), and mutations in these domains of histones H3 and H4 confer nucleosome instability (KURUMIZAKA and WOLFFE 1997; WECHSER *et al.* 1997). L1 of yeast histone H2B is also predicted to participate in internucleosomal interactions that might contribute to chromatin compaction (WHITE *et al.* 2001). Since the presence of nucleosomes influences the formation of UV-induced lesions (LIU *et al.* 2000; URA *et al.* 2001), we thought it likely that the H2B mutations might increase the overall
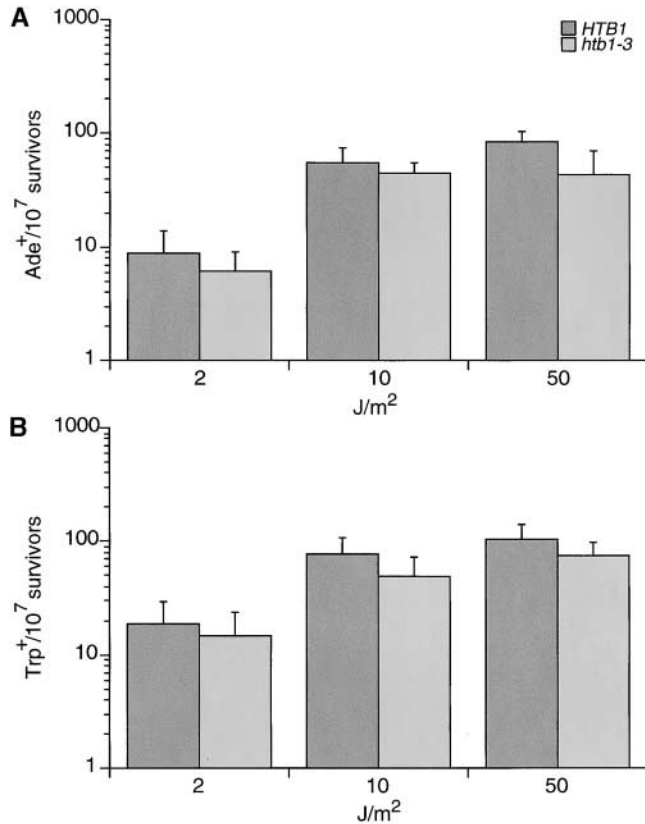
FIGURE 9.—Effect of the *htb1-3* allele on UV-induced mutagenesis. Reversion of the *ade2-1* and *trp1-1* mutations was measured in strain EM1 containing pRS314-*HTB1* or pRS314-*htb1-3*. The frequencies of Ade+ (A) or Trp+ (B) revertants per 10^7 survivors are shown as a function of UV dose. The data represent the mean ± standard deviation from two or more independent experiments. Frequencies of spontaneous UV-independent revertants were subtracted.

FIGURE 10.—A model for interactions in the *RAD6/RAD18*-dependent pathway of PRR. See text for discussion.

accessibility of chromatin to DNA-damaging agents. However, we found no evidence that nucleosomes were less stable in the *htb1-3* mutant or that the formation of CPD lesions was increased in this strain. This suggests that the particular amino acid changes in L1 and L2 of H2B do not lead to global chromatin opening, although we cannot exclude the possibility that they cause local changes in chromatin structure that influence lesion formation at discrete sites. Excision of CPDs and 6-4 photoproducts by the NER pathway is also strongly inhibited by the presence of nucleosomes (LIU and SMERDON 2000; URA *et al.* 2001). Thus, a lack of nucleosome mobility in the *htb1-3* mutant could interfere with the repair process itself. However, if this were the case, we would have expected the *htb1-3* mutation to be epistatic with mutations in NER, which was not observed. It therefore appears that the *htb1-3* allele does not significantly affect either the formation or the excision of UV-induced lesions on a global level.

H2B is in a novel *RAD5*-dependent branch of *RAD6/RAD18*-dependent postreplication repair: Genetic epistasis studies showed that the *htb1-3* allele affects the
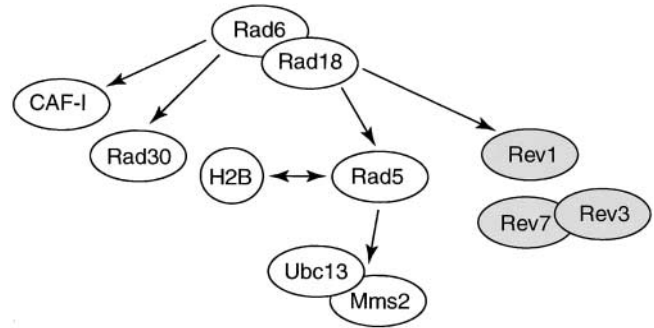
RAD6/RAD18-dependent PRR pathway, which corrects DNA lesions through both error-free and error-prone functions. Moreover, a *rad5Δ* mutation was epistatic to the *htb1-3* mutation (Figure 8A), placing H2B in a *RAD5*-dependent DNA repair pathway. The role of *RAD5* in PRR is unclear, although recent genetic and biochemical studies suggest that one function of Rad5p is to recruit the Ubc13p/Mms2p ubiquitin-conjugating complex to chromatin (ULRICH and JENTSCH 2000). This and other genetic evidence have led to the proposal that Rad5p-Ubc13p-Mms2p represents a distinct branch of PRR (ULRICH and JENTSCH 2000). Since H2B is a major chromatin constituent, we anticipated that it might also act in this branch. However, the *htb1-3* and *ubc13Δ* mutations were additive for UV sensitivity (Figure 8D), suggesting that H2B is involved in a novel *RAD5*-dependent branch of PRR that is separate from the Ubc13p-Mms2p branch (Figure 10).

H2B and chromatin assembly factor I act in distinct repair pathways: CAF-I was previously shown to play a role in the repair of UV-induced lesions (KAUFMAN *et al.* 1997; GAME and KAUFMAN 1999), and in this study we show that histone H2B also helps to protect against damage induced by UV. While both H2B and CAF-I act in PRR, it is likely that they perform different functions in this pathway. First, a *cac1Δ htb1-3* mutant was more sensitive to UV than either single mutant, indicating that CAF-I and H2B act in different branches of PRR. Second, CAF-I mutations enhance the UV sensitivity of mutations in all the major PRR genes except *RAD6* and *RAD18* (GAME and KAUFMAN 1999), whereas the *htb1-3* mutation is epistatic with mutations in *RAD5* as well as in *RAD6* and *RAD18*. Third, CAF-I interacts exclusively with histones H3 and H4 in its assembly function (SMITH and STILLMAN 1991b).

Distinguishing additive from synergistic effects of combining mutations in DNA repair factors: The observation of an additive effect when two null mutations are combined suggests that the genes involved affect different pathways acting on different lesions, whereas a synergistic effect suggests that the genes either belong in the same pathway or belong in different pathways

that work on the same lesion (Haynes and Kunz 1981). Previous work described a synergistic increase in UV sensitivity when the *rad5Δ* mutation was combined with either the *rev1Δ* or *rad30Δ* mutation (Johnson *et al.* 1992; McDonald *et al.* 1997; Xiao *et al.* 2000). On the basis of this observation, *RAD5* and *RAD30* have been proposed to be involved in the same pathway or in two overlapping pathways (McDonald *et al.* 1997). *mms2Δ* and *ubc13Δ* mutations show an epistatic relationship with *rad5Δ* in response to UV irradiation but an additive increase in UV sensitivity when combined with the *rad30Δ* mutation (Ulrich and Jentsch 2000). These results suggest that Rad30p and the Ubc13p-Mms2p complex are involved in two completely different sub-branches of the PRR pathway and also support the idea that Rad5p plays a dual role during the repair of UV-induced lesions (Cejka *et al.* 2001).

In this study, we show that (1) like *rad5Δ*, *htb1-3* shows a synergistic increase in UV sensitivity when combined with *rad30Δ*; (2) like *rad30Δ* but in contrast to *rad5Δ*, *htb1-3* shows an additive increase in UV sensitivity when combined with *ubc13Δ*; and (3) an *htb1-3 rad30Δ rad5Δ* triple mutant is not more UV sensitive than a *rad5Δ rad30Δ* double mutant (data not shown). Because *htb1-3* is a hypomorphic allele rather than a null mutation, the distinction between synergistic and additive effects has to be interpreted with caution. However, taken together, these results support the idea that H2B is involved with Rad5p in a UV-induced DNA repair pathway that is independent of the Ubc13p-Mms2p complex but related to Rad30p.

Effects of *htb1-3* on UV-induced mutagenesis: Earlier analysis of spontaneous and UV-induced mutagenesis at various loci performed in *rad5Δ* or *rad30Δ* single mutants did not specifically place *RAD5* and *RAD30* in the error-prone or error-free sub-branches of the PRR pathway. Instead, the combination of results obtained from double and triple mutants suggested a role for these two genes principally in the error-free subbranch of the PRR pathway, with a minor role in the error-prone pathway (Johnson *et al.* 1992; McDonald *et al.* 1997; Roush *et al.* 1998). In this work, we show that the *htb1-3* mutation alone did not significantly alter UV-induced reversion at two different loci and did not increase the reversion frequency when combined with the *rad5Δ* mutation (Figure 9 and data not shown). This is in contrast to what has been observed for the *rad5Δ rad30Δ* double mutant, which exhibits an elevated frequency of UV-induced mutation (McDonald *et al.* 1997). These results reinforce the idea that H2B and Rad5p could be involved together in a specific sub-branch of UV-induced DNA repair. However, we cannot determine whether the *htb1-3* mutation affects the error-free or error-prone pathway or both pathways.

What is the relationship between Rad5p and H2B? Rad5p contains several structural motifs that have been implicated in a number of biological processes. A RING finger motif in the C-terminal half of the protein has been shown to be necessary for its interaction with Ubc13p (Ulrich and Jentsch 2000). Rad5p also contains an ATPase domain with homology to the Swi2p/Snf2p family of ATPases (Hirschhorn *et al.* 1992; Johnson *et al.* 1992; Schild *et al.* 1992; Cairns *et al.* 1996; Du *et al.* 1998; Papoulas *et al.* 1998; Pollard and Peterson 1998). Several proteins that contain this ATPase domain have direct roles in destabilizing nucleosomes, a function that is important for transcriptional activation (Muchardt and Yaniv 1999; Peterson and Workman 2000). If Rad5p is also able to remodel nucleosomes, this activity might be important for some aspects of PRR. We suggest that H2B might play a role in chromatin remodeling by Rad5p, perhaps to facilitate the chromatin association or activity of both mutagenic and error-free repair polymerases.

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